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Technical Note: Sex Determination of Prehistoric Human Paleofeces

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ABSTRACT Analysis of 12 prehistoric human paleofecal specimens from the Mammoth Cave System, Kentucky has produced the first estimate of biological sex using fecal material from ancient humans. Accelerator mass spectrometry (AMS) radiocarbon dating indicates that the specimens range in age from ca. 2700 B.P. to 2300 B.P. Dietary contents and steroids were extracted and analyzed. Chromatography and radioimmunoassay were used to measure levels of testosterone and estradiol in both modern fecal reference samples and in ancient feces. Results indicate that all 12 paleofeces were probably deposited by males whose diet included a variety of native crops and wild plants. These preliminary analyses have the potential to revolutionize the investigation of gender difference in diet, health, and nutrition.

Fecal material contains the most direct evidence of prehistoric human diet and nutrition in the form of undigested food remains consumed by individuals (Reinhard and Bryant, 1992; Sobolik and Gerick, 1992). Since the first paleofecal analyses were conducted (Jennings, 1957; Jones, 1936; Webb and Baby, 1957; Young, 1910), researchers have focused on determining diet of populations rather than on questions related to individuals within the group (Sobolik, 1994), mainly because of the lack of techniques needed to document variability at the individual level. The potential of one such technique, fecal steroid analysis, is demonstrated by our determination of biological sex of depositor for 12 paleofecal samples. Fecal steroid analysis has proven to be a promising method for the study of endocrine function and hormone metabolism in modern humans (Aldercreutz and Martin, 1976) and nonhuman primates (Shideler et al., 1993; Strier and Ziegler, 1994; Wasser et al., 1988), and has proven useful in sexing monomorphic birds (Tell and Lasley, 1991). The goals of this study were to validate fecal steroid techniques for use in modern human samples, determine a hormone ratio that could be used to distinguish male samples from female samples, estimate the sex of paleofecal samples, and assess variability in diet between females and males.

Mammoth Cave and Salts Cave are part of the Mammoth Cave system, an extensive karstic formation located in west-central Kentucky (Fig. 1). Prehistoric mineral min-

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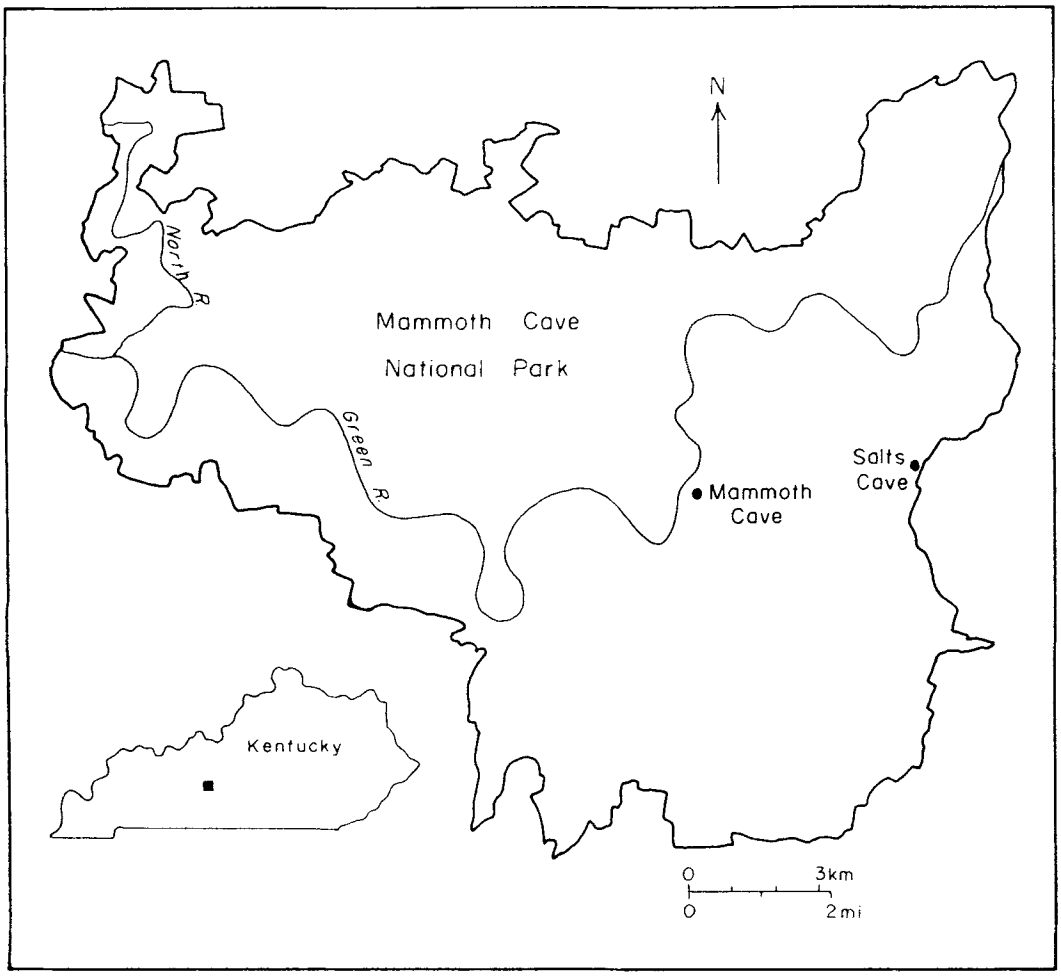

Fig. 1. Map of Mammoth Cave National Park showing location of Mammoth Cave and Salts Cave.

ing activity within these caves was most intensive between ca. 2400 and 2900 B.P. Near-constant temperature and other atmospheric conditions in upper passages have inhibited decay of prehistoric organic materials, including textiles, containers, torch remains, and paleofeces (Crothers and Watson, 1993). For this reason, archaeological material from interior passages of Salts Cave and Mammoth Cave has provided crucial evidence for the development of indigenous agricultural systems based on native crop plants in eastern North America. Analysis of paleofeces from these two caves demonstrated that native crops were a significant component of the cavers' diet as early as ca. 2700 B.P. (Watson, 1974; Yarnell, 1969, 1993). Further support for a pattern of reliance on garden crops and weeds has been obtained through examination of carbonized plant remains from the vestibule of Salts Cave (Gardner, 1987; Yarnell, 1974).

MATERIALS AND METHODS

Sample collection and processing

Paleofecal samples were collected from various locations in Salts Cave and Mammoth Cave. After removal of small amounts of material for radiocarbon dating at the NSF–Arizona AMS Facility, University of Arizona, the samples were taken to the Pa-

leoethnobotanical Laboratory at The Ohio State University for processing, dissemination to other researchers, and analysis. One half of each sample was immersed in 0.5% tri-sodium phosphate solution to disaggregate the contents (Callen and Cameron, 1960). The material was then passed through nested geological screens; material caught in the screens was analyzed for macrobotanical content at Ohio State, and material that passed through the screens was analyzed for pollen content at The Laboratory for Quaternary Paleoecology at The University of Maine.

Fresh samples were obtained from a modern reference group composed of four adult males and four adult females aged 20–50. Two of the female samples were obtained from the early follicular phase (days 5–7), and two were obtained from the luteal phase (day 21). A portion of each unimmersed paleofecal half was analyzed for steroid content at The Laboratory of Reproductive Ecology at Emory University and compared to modern reference samples.

A 0.1 g sample was homogenized in 3 ml methanol:acetone (8:2, v/v). The homogenate was filtered by layering onto a 0.2 mm G Prep nylon centrifuge filter (Gelman Sciences, Ann Arbor, MI) and centrifuging at 1,500 × *g* for 5 min. The filtrate was extracted using small columns of reversed-phase octadecylsilane (C18) bonded to silica (Sep-Pak C18, Waters Associates Inc., Milford, MA). The sample was layered onto the column, primed according to the manufacturer's directions. The column was then washed with 5 ml water, and the steroid fraction was eluted with 3 ml methanol.

Radioimmunoassay (RIA)

The estradiol (E_2) RIA followed microassay procedures of Worthman et al. (1990) using reagents from the Pantex direct ^{125}I Estradiol 174M kit. Sensitivity of the assay is 0.4 pg/ml. Working buffer was 0.1% gelatin phosphate–buffered saline (pH 7.4). The methanolic extract was evaporated under nitrogen and reconstituted 1:10 (modern samples) or 5:1 (paleosamples) in working buffer. ^{125}I E_2 tracer (20 μl) and antiserum (500 μl, diluted 1:8) were added to aliquots (50 μl) of the standards (diluted 1:10 to give concentrations of 1–300 pg/ml), samples, and controls (diluted 1:10). After overnight incubation at room temperature, a second antibody (500 μl, diluted 1:4) was added, and the incubates were vortexed, incubated 1 hr at room temperature, and centrifuged at 1,500 × *g* for 1 hr at room temperature. The supernatant was decanted, and the radioactivity in the precipitates was determined by 10 min counts in a gamma counter.

The testosterone (T) RIA followed the microassay procedures of Beall et al. (1992) using reagents from the Equate Testosterone RIA kit (Binax, South Portland, ME). Sensitivity of the assay is 6 pg/ml. Working buffer was 0.1% gelatin phosphate–buffered saline (pH 7.4). The methanolic extract was evaporated under nitrogen and reconstituted 1:10 (modern samples) or 5:1 (paleosamples) in working buffer. ^{125}I T tracer (50 μl) and antiserum (100 μl, diluted 1:2) were added to aliquots (100 μl) of standards (diluted 1:10 to give concentrations of 1–100 ng/dcl), samples, and controls (diluted 1:10). Each was vortexed and incubated overnight at room temperature. The second antibody (500 μl, diluted 1:2) was added, and the incubates were vortexed and centrifuged at 1,500 × *g* for 20 min at room temperature. Following decanting of the supernatants, radioactivity of the precipitate was determined by 10 min counts in a gamma counter.

Steroid assay validations

Previous investigations indicated that RIAs for T and E_2 produce accurate and reliable results when applied to the assay of primate fecal samples (Stavisky et al., 1995; Brockman et al., 1995). Further tests in human samples demonstrated their accuracy and reliability in these samples as well. Tube accuracy was 1.7% for E_2 and 1.2% for T. Method accuracy, tested by the recovery of steroid standards added in duplicate to fecal extracts, was 100.9 ± 5.6% for E_2 ($r = 0.999$; $P < 0.001$; $y = 0.94x + 2.91$; $n = 6$) and 137.6 ± 10.3% for T ($r = 0.999$; $P < 0.001$; $y = 0.84x - 0.79$; $n = 6$). Serial dilutions of fecal extracts yielded binding coefficients that paralleled standard curves for E_2 and T. Within-assay precision was tested by replicate determinations of high and low samples within the same assay. Intra-assay coef-

ficients of variation (CV) were 4.2–5.8% for E_2 and 9.8% for T. Interassay CVs, tested by replicate determinations of serum controls across assays, were 7.2–7.5% for E_2 and 3.3–5.7% for T.

High-performance liquid chromatography (HPLC)

HPLC was used to separate steroid metabolites in fecal extracts for tests of assay specificity (Brockman et al., 1995). Chromatography was conducted using a Perkin–Elmer (Norwalk, CT) HPLC system and a Hypersil ODS column (5 μm, 25 cm × 4.6 mm I.D., Aldrich, Milwaukee, WI). Estrogens were separated using an isocratic program (Setchell et al., 1987) with a mobile phase of methanol −0.1 M ammonium acetate buffer, pH 4.6 (60:40), with a flow rate of 1 ml/min at 30 C and UV detection at 280 nm. Androgens were separated by a gradient method (O'Hare et al., 1976) using an acetonitrile:water gradient of 60–80% with a flow rate of 1 ml/min at 45 C and UV detection at 240 nm. An aliquot of fecal extract was concentrated 10:1 (modern samples) or 150:1 (paleo samples), and a 20 μl sample was injected in mobile phase. The chromatographic eluent was collected in 0.3 min fractions, the fractions were reconstituted in assay buffer, and immunoreactivity was assessed in E_2 and T RIAs.

RESULTS

One of the goals of the study was to assess the dietary variability between males and females. Because all of the paleofecal samples were probably deposited by males, this goal cannot be achieved with this analysis. However, dietary analysis of the samples, including macrobotanical and pollen contents, indicate that prehistoric explorers of Salts Cave and Mammoth Cave ate a variety of plant foods, including substantial quantities of crops such as squash *(Cucurbita pepo),* chenopod *(Chenopodium berlandieri),* sunflower *(Helianthus annuus),* and sumpweed *(Iva annua),* in addition to a variety of wild plant foods (Fig. 2). An extensive dietary analysis is reported elsewhere (Gremillion and Sobolik, 1996).

Steroid detection and authentication

Measurable quantities of E_2 and T were detected in the paleofecal samples. E_2 concentrations ranged from 0.006 to 0.4 ng/g, and T concentrations ranged from 0.9 to 11 ng/g, dry weight. These values were all below the modern range of concentrations (see Table 1), suggesting that some steroid deterioration had occured since deposition. If there had been no loss over time, concentrations in modern samples, which are expressed per gram of wet weight, should have exceeded concentration in the paleosamples, which of necessity are dry weight determinations.

Because other steroid metabolites present in fecal extracts may crossreact with steroid antibodies, it was necessary to test the identity of material quantified by RIA. To do so, androgen and estrogen metabolites were first separated using HPLC, and chromatographic fractions were analyzed for immunoreactivity to determine contributions of steroids and steroid metabolites to total immunoreactivity in paleo and modern extracts. Figures 3 and 4 demonstrate that steroid concentrations estimated by RIA represent authentic E_2 and T. Only a single immunoreactive peak is detected by the E_2 antibody, corresponding to the retention time of E_2, in both modern (Fig. 3, top and middle) and paleo samples (Fig. 3, bottom). The T antibody detects an additional peak, corresponding to the retention time of the androgen metabolite androsterone, in two modern male samples (Fig. 4, top), but only a single peak, corresponding to the retention time of T, is evident in the extracts of modern females (Fig. 4, middle) and paleo samples (Fig. 4, bottom).

Sex differences in steroids

Examination of the modern range of values indicates that generally E_2 concentrations were higher in female extracts and T concentrations were higher in male extracts (see Table 1). The paleo samples exhibited a pattern of values reminiscent of modern reference males, with high T and low E_2 (see Fig. 2). However, evidence for steroid deterioration suggests that a ratio would be more useful than absolute values in distinguishing sex. Ratios of T to E_2 in modern males

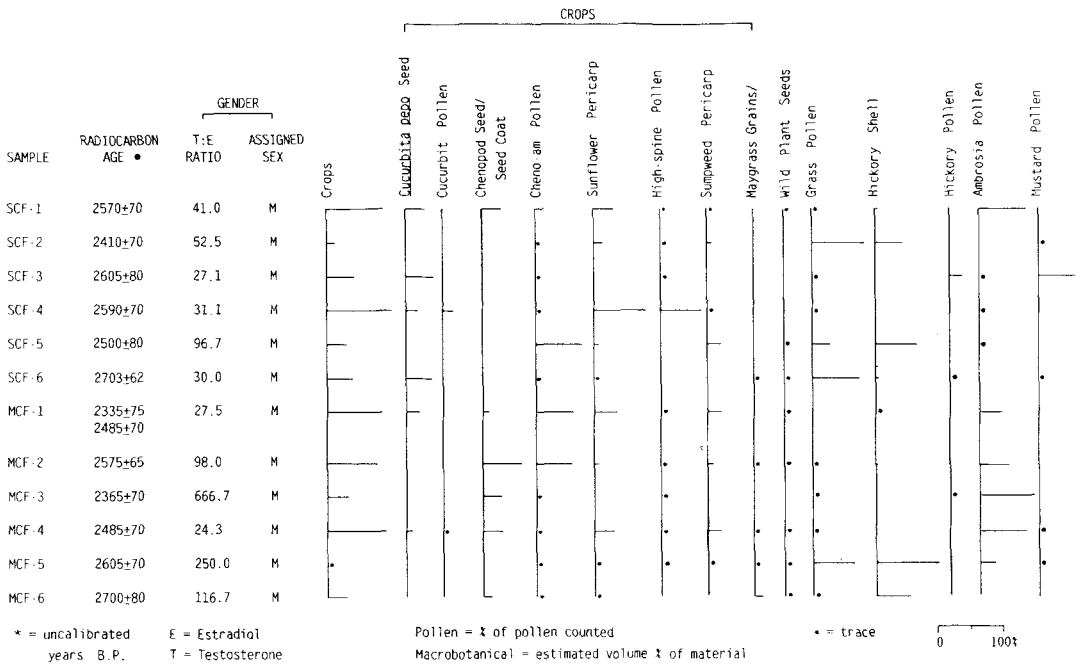

Fig. 2. Macrobotanical, pollen, and steroid content of paleofeces with associated radiocarbon determinations.

TABLE 1. Modern human fecal testosterone (T) and estradiol (E) concentrations

ID	Sex	Cycle day	T ng/g	E ng/g	T:E ratio
1	M		33.0	11.4	2.9
2	M		175.9	6.8	26.6
3	M		24.7	7.2	3.4
10	M		566.2	4.8	117.6
Mean			200.6	7.6	37.6
4	F	21	7.2	34.8	0.2
6	F	5	24.3	3.4	7.0
6	F	21	33.1	43.5	0.8
7	F	7	16.5	19.0	1.0
Mean			20.3	25.2	2.3

and females are given in Table 1. The ratio was higher in males, where it ranged from 3 to 118, than in females, where it ranged from 0.2 to 7, although there was some overlap of the follicular phase values of females with lower male values. All related Mammoth Cave and Salts Cave samples exhibited ratios of 24 or greater (Fig. 2). These values were significantly different from modern female values (Mann–Whitney rank sum test, $P = 0.004$) but did not differ significantly from modern male values ($P = 0.130$), suggesting that these samples were deposited by males.

DISCUSSION

These investigations demonstrate that E_2 and T can be reliably and accurately measured in fecal extracts from modern humans. The microassay procedures employed here also have enabled us to quantify small concentrations of steroids remaining in prehistoric human samples. Moreover, chromatographic and immunoassay procedures indicate that hormones measured in paleofecal samples are authentic E_2 and T. Because these steroids are present in much lower concentrations in paleofecal samples than in modern samples, it is likely that steroid decomposition has occurred over time, perhaps as a result of fecal bacteria and microbial pathogens present in the depositional environment.

The ratio of T:E_2 appears to be a useful index for differentiating male and female samples. Fecal ratios reported for modern reference samples are in accord with serum

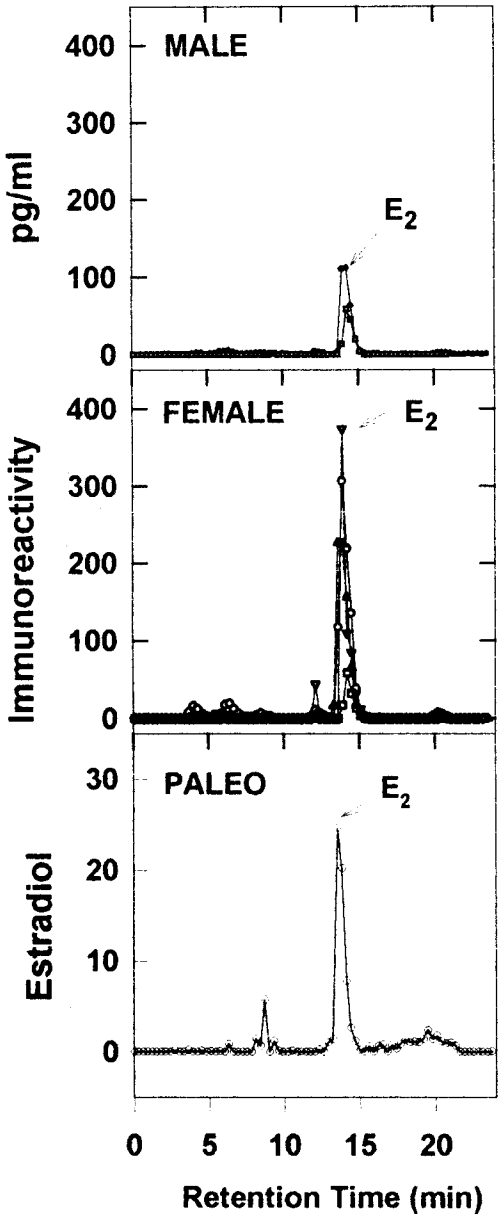

Fig. 3. Immunoreactivity of the E_2 antibody with chromatographic fractions collected from extracts of modern reference samples of males (top), females (middle), and paleosamples (bottom). The legend marks the retention time of the E_2 standard.

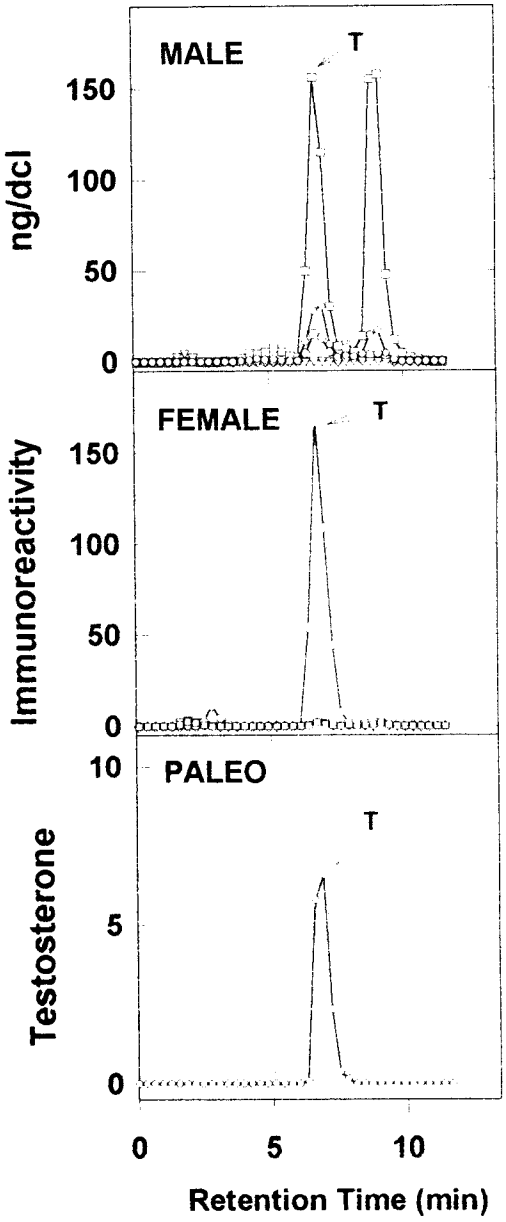

Fig. 4. Immunoreactivity of the T antibody with chromatographic fractions collected from extracts of modern reference samples of males (top), females (middle), and paleosamples (bottom). The legend marks the retention time of the T standard.

T:E_2 ratios in modern humans. T:E_2 ratios calculated from serum concentrations reported for Kalahari hunter-gatherers by Worthman (1978) range from 0.6 to 7 in females and from 47 to 376 in males. Serum ratios also are higher for women in the follicular phase (mean = 2.7) than in the ovulatory phase (mean = 1.6) or luteal phase (mean = 1.8) of the cycle.

High ratios of most paleofecal samples

suggest that these samples belong to males. Some caveats are in order, however. First, we have made the simplifying assumption that both steroids have declined at equivalent rates over time. However, the stability of the two steroids over long periods of time has not been documented, particularly in the rather unique environment of these caves. A more rapid decay rate for E_2 would enlarge the T:E_2 ratio, resulting in misclassification of some samples. One solution would be to estimate the half-lives of the two steroids and use them to correct the ratios if necessary. An additional caveat is the current small size of the modern reference sample. Our preliminary data indicate that female ratios vary with menstrual cycle and male values vary with age. A larger sample of individuals at varying ages and reproductive states would provide a better estimate of normal interindividual variation.

These preliminary data are promising, however, in demonstrating that it is possible to make determinations of sex in these prehistoric samples. The approach makes possible the analysis of gender-specific activities and diet in prehistoric sites. All samples analyzed here were probably deposited by males, which may indicate that only males were exploring Mammoth and Salts Caves during this period and that they were eating crop foods supplemented by wild plants. In other paleofecal analyses it may be possible to identify samples from both males and females, which could be used to assess gender differences in dietary intake. These investigations demonstrate that the technology and preliminary research necessary to document food consumption within groups of prehistoric populations are now available, offering the prospect of identifying gender-related patterns of dietary variability in prehistory.

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